

## Analysis for a Limited Number of Gene Codons Can Predict Drug Resistance of *Mycobacterium tuberculosis* in a High-Incidence Community

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**Correct and rapid diagnosis is essential in the management of multidrug-resistant tuberculosis (MDR-TB). In this population-based study of 61 patients with drug-resistant tuberculosis, we evaluated the frequency of mutations and compared the performance of genotypic (mutation analysis by dot blot hybridization) and phenotypic (indirect proportion method) drug resistance tests. Three selected codons (*rpoB531*, *rpoB526*, and *katG315*) allowed identification of 90% of MDR-TB cases. Ninety percent of rifampin, streptomycin, and ethambutol resistance and 75% of isoniazid resistance were detected by screening for six codons: *rpoB531*, *rpoB526*, *rrs513*, *rpsL43*, *embB306*, and *katG315*. The performance (reproducibility, sensitivity, and specificity) of the genotypic method was superior to that of the routine phenotypic method, with the exception of sensitivity for isoniazid resistance. A commercialized molecular genetic test for a limited number of target loci might be a good alternative for a drug resistance screening test in the context of an MDR “DOTS-plus” strategy.**

The emergence of drug-resistant strains of *Mycobacterium tuberculosis*, especially multidrug-resistant (MDR) strains, defined as resistant to at least isoniazid (INH) and rifampin (RIF) (15), poses a threat to the success of tuberculosis (TB) control programs. As a consequence of the increase in MDR TB (MDR-TB) and the relatively restricted number of therapeutic agents, there has been a renewed effort during the last decade to define the molecular basis of drug resistance in *M. tuberculosis*. Resistance to drugs is due to particular genomic mutations in specific genes of *M. tuberculosis* (17). To date, nine genes are known to be linked to resistance to first-line anti-TB drugs: *katG*, *inhA*, *aphC*, and *kasA* for INH resistance; *rpoB* for RIF resistance; *rpsL* and *rrs* for streptomycin (STR) resistance; *embB* for ethambutol (EMB) resistance; and *pncA* for pyrazinamide resistance. Resistance to multiple drugs is the consequence of an accumulation of mutations (8, 13).

Under the current World Health Organization guidelines for TB control in low- and middle-income countries (11), diagnosis of new TB patients is based on examination of sputum smears by microscopy for the presence of acid-fast organisms. Cases of primary drug-resistant TB thus will be missed, with consequent prolonged infectivity and further spread of drug-resistant TB. A new strategy, “DOTS plus” (4, 7), which includes *M. tuberculosis* culturing and sensitivity testing at diagnosis, has been introduced in a few pilot projects. However, when drug susceptibility testing is culture based, detection still takes 2 to 9 weeks (7). The molecular basis of drug resistance

in *M. tuberculosis* makes it possible to create new, rapid diagnostic tests. Rapid detection of drug resistance not only could optimize treatment and improve outcome for patients with drug-resistant TB but also is especially important in the prevention of transmission of drug-resistant TB. When the first study on detection of mutations in clinical isolates was published, it was hoped that early detection of resistance in *M. tuberculosis* would soon be routine clinical practice (19). Seven years later, mutation detection analysis is still not part of clinical practice. To be cost-effective in resource-poor countries, where most MDR-TB patients reside, it is crucial that molecular genetic tests fulfill the criteria of accuracy, speed, and simplicity. Evaluation of the frequency distribution of various mutations in clinical isolates originating from different geographical regions is essential for the selection of a limited number of target mutations to enable the detection of the majority of drug resistance (3, 16).

In this study, we investigate the frequency of gene mutations in clinical isolates of *M. tuberculosis* originating from two communities of metropolitan Cape Town (Western Cape Province, South Africa) with a high incidence of TB and documented outbreaks of MDR-TB (22). This area provides the possibility of comparing the clinical usefulness of a genotypic method to that of a culture-based phenotypic drug susceptibility test under routine conditions.

### MATERIALS AND METHODS

**Setting.** The patients described in this paper were identified as having active cases of drug-resistant TB (on the basis of culture-based drug susceptibility testing) between 1 April 1992 and 31 March 1997. All patients resided in two neighboring communities of metropolitan Cape Town, a 2.4-km<sup>2</sup> area with a population of approximately 34,000 people living in poor socioeconomic conditions. The rate of new bacteriologically confirmed cases in these communities is

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225/100,000/year (21). A survey of drug-resistant TB in Western Cape Province conducted in 1992 to 1993 found rates of 8.6% acquired and 3.2% initial drug resistance in the region (25). The reported prevalence of human immunodeficiency virus (HIV) infection in the region ranged from 0.25% in 1992 to 3% in 1996 (data from national HIV surveys of women attending antenatal clinics, conducted by the Department of Health and Population Development, Cape Town, Western Province, South Africa).

All patients were treated by direct observation at local primary health care clinics; 62% also received inpatient care. Compliance during treatment was defined as the intake of >80% of prescribed dosages before interruption or completion of treatment.

**Laboratory procedures.** Sputum samples were sent for microscopic examination and culturing to a provincial reference laboratory for drug susceptibility testing. Phenotypic drug susceptibility testing was performed by the economical version of the indirect proportion method (10) with Lowenstein-Jensen medium containing critical concentrations of 0.2 µg of INH, 30 µg of RIF, 2 µg of EMB, 5 µg of STR, and 20 µg of ethionamide per ml. Resistance was defined as 1% or more bacterial growth in comparison with a control, using international criteria.

Genotypic drug resistance testing was performed by mutation analysis according to a recently described PCR-based dot blot method (23). Specially designed primers (for regions in genes known to confer resistance in *M. tuberculosis*) were used to amplify genomic DNA extracted from clinical isolates of *M. tuberculosis*. Efficient PCR amplification was confirmed by gel electrophoresis. An aliquot of each PCR product was denatured and fixed on a Hybond-N<sup>+</sup> membrane by use of a dot blot apparatus (Bio-Rad). Discrimination between wild-type and mutant sequences was obtained under stringent hybridization conditions with labeled wild-type and mutant probes, respectively. The probes used in this study were directed toward mutations most frequently described in the literature. All samples were tested for mutations at the following codons: *katG315*, *kasA269*, *inhA10* (putative promoter), *inhA34* (putative promoter), *rpoB531*, *rpoB526*, *rpoB516*, *rpsL 43*, *rpsL88*, *rrs-513*, *rrs-491*, and *embB306*. Reference strain H37Rv, 10 fully susceptible isolates, and isolates characterized by gene sequencing as mutant (resistant) or wild type (susceptible) for specific gene codons were used as negative and positive controls, respectively. When resistance could not be explained by the identification of mutations in the above gene codons, samples were also tested for mutations in additional codons (*katG 275*, *katG409*, *kasA66*, *kasA312*, *kasA413*, *inhA15* (putative promoter), *rpoB533*, *rpoB513*, and *rrs-904*). Direct sequencing of selected PCR products was performed with a Sequenase PCR product sequencing kit (United States Biochemical Corp., Cleveland, Ohio) according to the manufacturer's instructions.

*M. tuberculosis* isolates were also genotyped by restriction fragment length polymorphism (RFLP) analysis (20). A cluster was defined as a group of two or more isolates originating from different patients and whose RFLP fingerprint patterns were identical with respect to both the number and the molecular size of all bands (1).

For each patient, the first and last available isolates were defined as the first and last isolates for which DNA was available for mutation detection and RFLP analysis. The results for the first available isolates were used to evaluate the frequency of mutations and thereby to compare the clinical usefulness of phenotypic and genotypic methods. The results for the last available isolates were used to determine the acquisition of additional mutations during treatment.

A discrepancy between the results of the phenotypic and genotypic drug resistance tests for the first available isolate was defined as a possible false-positive result for the genotypic method if the isolate was predicted to be resistant by mutation analysis but phenotypically drug susceptible. A false-negative result for the genotypic method was defined as a phenotypically resistant isolate in which no mutations conferring resistance were detected.

In cases of discrepancies, the *M. tuberculosis* isolate was retested by the phenotypic and genotypic methods if no follow-up isolate was available. Retesting by the phenotypic method was performed using a different method (BACTEC) at a different laboratory (Department of Biochemistry, University of Stellenbosch). In cases of discrepancies in which at least one follow-up isolate was available, the results for the follow-up isolate(s) were used to reevaluate the discrepancy for the first isolate.

After investigation of all discrepancies, the "corrected pattern" for each of the first available isolates was determined using a method similar to that used by Telenti et al. (18). This corrected pattern was used as the "gold standard" to evaluate and compare intrinsic characteristics, such as the sensitivity and specificity of both phenotypic and genotypic drug resistance testing.

Results of drug susceptibility testing of consecutive isolates from individual patients were used to determine the reproducibility of both phenotypic and genotypic tests by calculating the kappa coefficient (5). Pairs of isolates retrieved either from a single clinical episode or from different clinical episodes but caused

by the same *M. tuberculosis* strain, as determined by RFLP analysis, were included in this analysis. Pairs of isolates where the first isolate was drug susceptible and the following isolate was drug resistant were discarded for this analysis, as this situation might represent not a lack of intrasubject variation but acquisition of resistance during treatment.

## RESULTS

**Patient characteristics.** Between April 1992 and March 1997, 70 patients were identified as having isolates phenotypically resistant to one or more anti-TB drugs. In 61 of these, a minimum of one culture was available for molecular genetic analysis. These 61 patients constitute the study population. All patients had pulmonary TB, and 84% were smear positive. About half of the patients were female (31, or 51%). The mean age at diagnosis of drug-resistant TB was 33 years (range, 11 to 55 years). Fifty-two patients (85%) were tested for HIV, and all were seronegative. Thirty-five patients (57%) were compliant during their MDR-TB treatment.

**Phenotypic resistance pattern of first available *M. tuberculosis* isolates.** The resistance pattern was determined by phenotypic drug susceptibility testing (Tables 1 and 2). For 34 patients (56%), the first isolate available for this study was the isolate for which the diagnosis of drug resistance was made. For two patients 41 and 42, resistance was detected by mutation analysis of an isolate predating the isolate for which the diagnosis of drug resistance was made by the phenotypic method. Resistance was detected in 57 patients (93%) for INH, in 34 (56%) for RIF, in 25 (41%) for STR, and in 11 (18%) for EMB. Thirty-two patients (52%) were diagnosed as having MDR-TB.

Additional resistance acquired during treatment was diagnosed in 40% ( $n = 25$ ) of the patients (Table 1). The additional resistance acquired was for INH ( $n = 2$ ), EMB ( $n = 8$ ), RIF ( $n = 10$ ), and STR ( $n = 14$ ).

**RFLP data.** Six clusters of drug-resistant strains and 23 unique strains were identified (Table 1). Cluster 1 (19 IS6110 insertion elements with a pattern resembling that of strain W [6]) and cluster 2 (5 IS6110 insertion elements) represent the predominant types of drug-resistant strains in the communities tested.

**Genotypic analysis of first available *M. tuberculosis* isolates.** Mutations in genes conferring resistance to INH were detected in 43 isolates (70%) (Tables 1 and 2). The most frequent mutation associated with INH resistance was at codon 315 of the *katG* gene (41 of 43, or 95%). One isolate (2%) had a mutation in *inhA15* (putative promoter), and one isolate had a mutation in *kasA269*. A *katG315* mutation was present in one of four isolates (25%) classified as phenotypically susceptible to INH. No mutations could be detected in 16 of 57 isolates (28%) classified as phenotypically resistant to INH.

Mutations in genes conferring resistance to RIF were identified in 41 isolates (62%) (Tables 1 and 2). Codon 531 of the *rpoB* gene was the location of the most frequent mutation associated with RIF resistance (34 of 41, or 83%). Other mutations were detected in *rpoB526* (3 of 41, or 7%), *rpoB516* (2 of 41, or 5%), *rpoB533* (1 of 41, or 2%), and *rpoB518* (1 of 41, or 2%). Nine (22%) of the 41 isolates with *rpoB* mutations were phenotypically classified as susceptible to RIF. No *rpoB* mutations could be detected in 2 (6%) of the 34 isolates phenotypically classified as RIF resistant.

TABLE 1. RFLP classification and phenotypic, genotypic, and corrected drug resistance patterns for isolates from 61 patients<sup>a</sup>

Patient	RFLP pattern	First isolate						Interval to last isolate (days)	Last isolate		Patient compliant
		Phenotypic resistance pattern	Mutation analysis				Corrected pattern		Additional resistance pattern	Additional mutations	
			INH	RIF	STR	EMB					
1	Cluster 1	H, R	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	323	S, E		Yes
2	Cluster 1	H, R, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	176	S		Yes
3	Cluster 1	H, R, S, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	399			No
4	Cluster 1	H, S	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	NA	R, E		Yes
5	Cluster 1	H, R, S, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	NA			Yes
6	Cluster 1	H, R, S, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513<sup>b</sup></i>	<i>embB306</i>	H, R, S, E	302			No
7	Cluster 1	H, R, S, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	175			No
8	Cluster 1	H, R, S, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	77			No
9	Cluster 1	H, R, S	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	346	E		Yes
10	Cluster 1	H, R	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	NA	S		Yes
11	Cluster 1	H, R, S, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	277			Yes
12	Cluster 1	H, R, S	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	NA	E		Yes
13	Cluster 1	H, R, S, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	NA			Yes
14	Cluster 1	H, R	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	269	S, E		No
15	Cluster 1	H, R, S, E	<i>katG315</i>	<i>rpoB531</i>	<i>rrs-513</i>	<i>embB306</i>	H, R, S, E	NA			No
16	Cluster 2	H	<i>katG315d</i>	<i>rpoB531<sup>b</sup></i>	<i>rpsL43<sup>b</sup></i>		H, R, S	363	R, S		Yes
17	Cluster 2	H, R, S	<i>katG315d</i>	<i>rpoB531</i>	<i>rpsL43<sup>b</sup></i>		H, R, S	NA			Yes
18	Cluster 2	H, R, S	<i>katG315d</i>	<i>rpoB531</i>	<i>rpsL43</i>		H, R, S	394			No
19	Cluster 2	H, R, S	<i>katG315d</i>	<i>rpoB531</i>	<i>rpsL43</i>		H, R, S	215			Yes
20	Unique	H, S	<i>katG315d</i>	<i>rpoB516<sup>b</sup></i>	<i>rpsL43</i>		H, R, S	1,026	R		No
21	Cluster 2	H, R, S	<i>katG315d</i>	<i>rpoB531</i>	<i>rpsL43</i>		H, R, S	NA			Yes
22	Cluster 2	H, R, S	<i>katG315d</i>	<i>rpoB531</i>	<i>rpsL43</i>		H, R, S	660	E		No
23	Cluster 2	H, S	<i>katG315d<sup>b</sup></i>	<i>rpoB533<sup>b</sup></i>	<i>rpsL43</i>		H, S	201			No
24	Cluster 2	H, R, S	<i>katG315d</i>	<i>rpoB531</i>	<i>rpsL43</i>		H, R, S	NA			Yes
25	Cluster 2	H, R, S	<i>katG315d</i>	<i>rpoB531</i>	<i>rpsL43</i>		H, R, S	NA			Yes
26	Cluster 2	H, R	<i>katG315d</i>	<i>rpoB531</i>	<i>rpsL43</i>		H, R, S	444	S		No
27	Cluster 3	H, R	<i>katG315</i>	<i>rpoB531</i>			H, R	NA			Yes
28	Cluster 3	H, R	<i>katG315</i>	<i>rpoB531</i>			H, R	1,450	S		Yes
29	Cluster 3	H, R	<i>katG315</i>	<i>rpoB531</i>			H, R	14			Yes
30	Cluster 4	H	— <sup>c</sup>				None	124	R	<i>rpoB526<sup>b</sup></i>	No
31	Cluster 4	H					H	493			Yes
32	Cluster 4	H, R		<i>rpoB531</i>	<i>rpsL43</i>		H, R, S	NA			No
33	Cluster 4	R		<i>rpoB531</i>			R	1,188	H	<i>inhA15</i>	No
34	Cluster 5	H, R, S	<i>katG315</i>	<i>rpoB531</i>	<i>rpsL43</i>	<i>embB306<sup>b</sup></i>	H, R, S, E	NA			Yes
35	Cluster 5	H, R	<i>katG315</i>	<i>rpoB526</i>	<i>rpsL43</i>		H, R, S	NA	S		Yes
36	Cluster 5	H, R, S	<i>katG315</i>	<i>rpoB526</i>	<i>rpsL43</i>		H, R, S	103			No
37	Cluster 6	H	<i>katG315</i>				H	NA			Yes
38	Cluster 6	H	<i>katG315</i>		<i>rrs-491</i>		H, S	332	S		Yes
39	Unique	H	— <sup>c</sup>	<i>rpoB518<sup>b</sup></i>			H	75			Yes
40	Unique	H					H	NA			No
41	Unique	None	<i>katG315d</i>	<i>rpoB531</i>			H, R	2,154	H, R, S		No
42	Unique	None		<i>rpoB531<sup>b</sup></i>			R	NA	R		No
43	Unique	H	<i>katG315</i>				H	NA			Yes
44	Unique	H, R, E				<i>embB306</i>	H, R, E	92	S	<i>rpoB526</i>	No
45	Unique	R	<i>inhA15<sup>b</sup></i>	<i>rpoB531</i>			R	NA			Yes
46	Unique	H, S	<i>katG315</i>				H, S	253	E		Yes
47	Unique	H					None	268		<i>rpoB516</i>	No
48	Unique	H					H	613	R, S	<i>rpoB531</i>	No
49	Unique	H					H	NA			No
50	Unique	H, R, E		<i>rpoB516<sup>b</sup></i>		<i>embB306</i>	H, R, E	683	S	<i>inhA34</i>	No
51	Unique	H					H	NA			Yes
52	Unique	H, S	<i>katG315</i>	— <sup>c</sup>			H, S	131	R	<i>rpoB526</i>	Yes
53	Unique	H					H	NA			Yes
54	Unique	H		<i>rpoB531</i>			H, R	307	R	<i>rrs-491</i>	No
55	Unique	H			<i>rrs-491</i>		H	669			Yes
56	Unique	H	<i>kasA269<sup>b</sup></i>				H	NA			No
57	Unique	H, R			<i>rrs-491</i>		None	NA			NA
58	Unique	H					H	NA			Yes
59	Unique	H	<i>katG315</i>	<i>rpoB526</i>	<i>rpsL43</i>	<i>embB306</i>	H, R, S, E	1,056	S, E, R		Yes
60	Unique	H	<i>katG315</i>				H	NA			Yes
61	Unique	H, S	<i>katG315d<sup>+</sup></i>		<i>rrs-513</i>		H, S	NA			No

<sup>a</sup> H, INH; R, RIF; S, STR; E, EMB. Mutation analysis for first isolates indicates the genes encoding resistance to the indicated drugs. *katG315*, AGC→ACC (Ser→Thr); *katG315d*, AGC→ACA (Ser→Thr); *katG315d<sup>+</sup>*, *katG315d* plus *inhA15* (C→T<sub>-15</sub>; putative promoter); *kasA269*, GGT→GAT (Gly→Ser); *rpoB531*, TCG→TTG (Ser→Leu); *rpoB526*, CAC→TAC (His→Tyr); *rpoB516*, GAC→GTC (Asp→Val); *rpoB518d*, AAC (Asn) deletion; *rpoB533*, CTC→CCC (Leu→Pro); *embB306*, ATG→GTG (Met→Val); *rpsL43*, AAG→AGG (Lys→Arg); *rrs-491*, C→T<sub>491</sub>; *rrs-513*, A→C<sub>513</sub>; *inhA34*, C→T<sub>-34</sub> (putative promoter). NA, not available. Mutations were confirmed using mutant probes in conjunction with the dot blot method.

<sup>b</sup> The presence of the mutation was confirmed by DNA sequencing.

<sup>c</sup> —, The absence of a specific mutation was confirmed by DNA sequencing.



TABLE 2. Number (percentage) of resistant first available isolates, as determined by different methods

Method	No. (%) resistant to:				
	INH	RIF	STR	EMB	INH + RIF
Phenotypic test	57 (93)	34 (56)	25 (41)	11 (18)	32 (52)
Genotypic test	43 (70)	41 (62)	35 (57)	19 (31)	34 (56)
Corrected	55 (90)	40 (66)	35 (57)	19 (31)	37 (61)

A total of 34 of the 61 isolates (56%) had mutations in both the *katG* and the *rpoB* genes. Seven of these isolates were not phenotypically classified as MDR. Mutations conferring resistance to both INH and RIF were absent in 4 of 32 isolates (12%) phenotypically classified as MDR.

Mutations in genes conferring resistance to STR were detected in 35 isolates (57%) (Tables 1 and 2). The most frequent mutations conferring resistance to STR were found at position 513 of the *rrs* gene (16 of 35, or 46%) and codon 43 of the *rpsL* gene (16 of 35, or 46%). Three isolates (8%) had a mutation at position 491 of the *rrs* gene. Mutations were present in 12 of the 36 isolates (33%) phenotypically classified as susceptible to STR. Mutations could not be detected in 2 of the 25 isolates (8%) phenotypically classified as STR resistant.

Codon 306 of the *embB* gene was the only codon screened for EMB resistance. A mutation was detected in 19 isolates (31%) (Tables 1 and 2). Mutations were present in 8 of 50 isolates (16%) phenotypically classified as susceptible to EMB. *embB306* mutations were detected in all 11 isolates phenotypically classified as resistant to EMB.

Except for one drug-susceptible control isolate with an *rrs-491* mutation, none of the other nine fully susceptible isolates or the H37Rv control strain had mutations in the codons screened.

**Investigation of discrepancies between phenotypic and genotypic tests of the first available *M. tuberculosis* isolates.** Twenty discrepancies (INH, 16; STR, 2; and RIF, 2) represented potential false-negative results of mutation analysis, according to the definition outlined above. Retesting by the genotypic method confirmed prior results in all cases. Ten of these isolates were confirmed resistant according to the results of a phenotypic test of follow-up isolates (INH, 7; STR, 2; and RIF, 1). Upon retesting of the remaining 10 isolates by the phenotypic method, 4 isolates were confirmed resistant to INH. Two isolates could not be retested, as they had lost viability. For further analysis, the original phenotypic test result for these two isolates was considered correct. Four isolates were found to be drug susceptible on retesting by the phenotypic method (INH, 3; and RIF, 1).

In conclusion, after investigation of the 20 discrepancies, 16 cases of false-negative results of mutation analysis remained. False-negative results occurred predominantly for INH resistance.

There were 31 (STR, 12; RIF, 9; EMB, 8; and INH, 2) potential false-positive results of mutation analysis, according to the definition proposed above. The presence of a mutation was confirmed by use of follow-up isolates ( $n = 21$ ) or upon retesting of the first isolate ( $n = 10$ ). Phenotypic resistance was detected with the next available follow-up isolate in 23 cases. The genotypic classification of the first isolate as resistant was

considered correct in the 23 cases where the mutation present in the first isolate conferred the resistance phenotypically detected in the follow-up isolate. Five isolates were classified as drug susceptible because follow-up isolates remained drug susceptible. The three remaining isolates were phenotypically retested. Isolate 10 was found to be resistant to EMB. Isolate 32 was found to be resistant to 2 and 4  $\mu$ g of STR but susceptible to 8  $\mu$ g of STR. Isolate 34 was found to be resistant to 2.5  $\mu$ g of EMB but susceptible to 5 and 10  $\mu$ g of EMB.

In conclusion, after investigation of the 31 discrepancies, 5 cases of false-positive results of mutation analysis remained. False-positive results occurred with rare mutations, such as *rpoB533*, *rpoB518d* (Table 1), *rrs-491*, and *inhA15* (putative promoter).

**Additional resistance acquired during treatment.** Additional resistance acquired during treatment (as determined by the phenotypic method) was present in 25 patients (40%) (Table 1). Of these patients, 46% ( $n = 12$ ) were non compliant during treatment. The mutation conferring the acquired additional resistance was found to be present in the first available isolate in 9 of 14 instances (64%) of additional resistance to STR, 7 of 11 instances (64%) of additional resistance to RIF, 6 of 8 instances (75%) of additional resistance to EMB, and 3 of 4 instances (75%) of additional resistance to INH.

For 35 patients, a follow-up isolate was available for genotypic analysis (Table 1). The time between the first and last isolates was, on average, 485 days (range, 14 to 2,145) days. The first and last available isolates possessed the same RFLP pattern in all cases. An additional mutation was detected in 8 (23%) of the 35 patients. Seven of these eight patients (88%) with an additional mutation on follow-up were noncompliant.

**Performance of phenotypic and genotypic tests.** After correction for errors in phenotype assessment and mutation analysis, the resistance pattern of each first available isolate was reclassified (Tables 1 and 2). Isolates were correctly classified as MDR in 90% of cases by the genotypic method and in 84% of cases by the phenotypic method. Phenotypic resistance was detected in 98, 83, 71, and 58% of all isolates resistant to INH, RIF, STR, and EMB, respectively, according to the correct classification. The diagnosis of resistance of the first isolate by the phenotypic method was thus missed for 2% of isolates ( $n = 1$ ) resistant to INH, 17% of isolates ( $n = 7$ ) resistant to RIF, 29% of isolates ( $n = 10$ ) resistant to STR, and 42% of isolates ( $n = 8$ ) resistant to EMB. Genotypic resistance was detected in 76, 98, 94, and 100% of all isolates resistant to INH, RIF, STR, and EMB, respectively, according to the correct classification. Detection of resistance was thus missed for 24% of isolates ( $n = 13$ ) resistant to INH, 2% of isolates ( $n = 1$ ) resistant to RIF, and 6% of isolates ( $n = 2$ ) resistant to STR. The lowest yield of mutation detection (31%) was for isolates resistant only to INH.

The reproducibility, sensitivity, and specificity of each method were evaluated for each drug using the corrected classification as the gold standard (Table 3). Genotypic analysis had a reproducibility of 100% (kappa value, 1.0) for all codons tested (no discrepancies between 151 follow-up isolates from 35 individual patients). The reproducibility of the phenotypic method was evaluated with 246 follow-up isolates from 54 patients and found to be fair for INH and EMB (respective kappa values, 0.43 and 0.49) and good for RIF and STR (re-

TABLE 3. Validity and reliability of phenotypic and genotypic drug resistance testing

Drug	Test	Reproducibility (Kappa coefficient)	%	
			Sensitivity	Specificity
INH	Phenotypic	0.43	98	50 <sup>a</sup>
	Genotypic	1	76	83 <sup>a</sup>
RIF	Phenotypic	0.68	83	95
	Genotypic	1	98	91
STR	Phenotypic	0.6	71	100
	Genotypic	1	94	92
EMB	Phenotypic	0.49	58	100
	Genotypic	1	100	100

<sup>a</sup> Results could not be interpreted correctly because of the high prevalence (90%) of INH resistance in the study population.

spective kappa values, 0.68 and 0.6). Except for INH, sensitivity (ability to detect true drug resistance) was lower for the phenotypic test, while specificity (ability to detect true drug susceptibility) was lower for the genotypic test.

## DISCUSSION

In this population-based study, we investigated 61 patients diagnosed with drug-resistant TB by conventional drug susceptibility methods. The analysis of the results focused on the first available isolate from each patient, as this is the most important isolate for patient management. We did not correct for the number of strains involved in an outbreak because the detection of resistance is important for clinical management regardless of the classification of an isolate as clustered or unique. In contrast to the practice in most studies, where phenotypic tests are performed in a high-quality national, supranational, or research laboratory, the phenotypic tests for this study were performed in a routine provincial laboratory to approximate the everyday reality of *M. tuberculosis* drug resistance testing in a middle-income country. As done previously by Telenti et al. (18), we did not use the phenotypic test as the gold standard but used the "corrected" version of resistance patterns obtained by investigation of discrepancies between phenotypic and genotypic tests. In this way, the performance of both phenotypic and genotypic tests could be evaluated and compared.

In this study, genotypic tests for only three selected codons (*rpoB531*, *rpoB526*, and *katG315*) allowed correct identification of 90% of all MDR-TB cases from the communities studied during the 5-year study period. Furthermore, more than 90% of resistance to RIF, STR, and EMB and 75% of resistance to INH could be detected by screening for only six codons: *rpoB531*, *rpoB526*, *rrs-513*, *rpsL43*, *embB306*, and *katG315*. Except for *rrs-513*, this array of codons is reported in the literature as the most frequently mutated loci (9, 12, 14, 16, 17, 26). The only category with a low yield of mutation analysis (31%) was for isolates resistant only to INH. The genotypic method was superior to the phenotypic method for the correct identification of resistance to RIF, STR, and EMB, while the phenotypic method was superior for the correct identification of INH resistance.

An important question in the evaluation of a new diagnostic

test is the advantages and disadvantages compared to those of existing tests. Sensitivity, specificity, and reproducibility are important elements in this comparison, besides complexity, labor-intensiveness, turnaround time for results, and cost. The reproducibility was excellent for the genotypic test and fair to good for the phenotypic test. Under such conditions, a phenotypic test will be evaluated as relatively unreliable by a health professional and repeated several times to ensure correct clinical management, while one can rely on a single positive molecular genetic test. This fact reduces the total time to diagnosis of a molecular genetic test even further compared to that of a phenotypic test. In this study, resistance would have been detected by a molecular genetic test prior to the phenotypic test for 2% of INH-resistant isolates, 17% of RIF-resistant isolates, 29% of STR-resistant isolates, and 42% of EMB-resistant isolates.

The poorer performance of the phenotypic test could serve as an explanation for the discrepancy between the acquisition of phenotypic resistance during therapy (25 cases) and the acquisition of an additional mutation (8 cases). This speculation is supported by the fact that 88% of patients acquiring an additional mutation while on treatment were noncompliant; in comparison, only 46% of patients with phenotypically acquired additional resistance were noncompliant.

The specificity of both methods was high for RIF, STR, and EMB resistance. The specificity for INH resistance could not be interpreted because of the extremely high prevalence (90%) of INH resistance in the population studied. The sensitivity (detection of true drug resistance) of the molecular genetic test was superior to that of the phenotypic test for the detection of resistance to RIF, STR, and EMB, while the sensitivity of the phenotypic test was superior for the detection of INH resistance.

There remain, however, major limitations to the molecular genetic detection of drug resistance (2). (i) Molecular genetic tests detect only mutations that are screened for, while phenotypic tests detect resistance independent of the underlying mechanism. (ii) Not all mutations conferring resistance to anti-TB drugs are known. This fact is especially a problem in the detection of INH resistance and explains the low sensitivity of the genotypic method for INH resistance testing. (iii) Only a few mutations conferring resistance to second-line drugs are known. (iv) The causal relationship between the presence of a mutation and the occurrence of resistance has been shown for some mutations, for example, codon 315 in the *katG* gene (24); however, a causal relationship has not been reported for all mutations currently believed to confer resistance. In our study, false-positive results were obtained with *rpoB533*, *rpoB518*, *rrs-419*, and *inhA15*, mutations that are infrequently described (17). It is possible that these mutations are silent or confer low-level resistance, making the diagnosis difficult. Analysis of the *rrs-491* mutation in this study showed that this mutation was also detected in several pansusceptible isolates. The mutation responsible for low-grade resistance to EMB in patient 34 was explained by a *embB306* mutation (ATG to GTG). Further research should therefore be directed at establishing causal relationships between specific mutations and drug resistance.

The high sensitivity of, the rapid diagnosis by, and the high reliability of genotypic drug resistance testing are important, as

they will allow appropriate patient management within days of TB diagnosis. The detection of 90% of cases of MDR-TB by screening of only three gene codons (*rpoB531*, *rpoB526*, and *katG315*), the correct identification of more than 90% of isolates resistant to RIF, STR, and EMB by screening of five gene codons (*rpoB531*, *rpoB526*, *rrs-513*, *rpsL43*, and *embB306*), and the correct identification of 75% of isolates resistant to INH by screening of one codon (*katG315*) are promising for the development of a cost-effective commercial screening test. Although molecular genetic testing cannot as yet (and probably will never) fully replace traditional phenotypic susceptibility testing, a commercialized molecular genetic test for a limited number of target codons might be a good alternative for a drug resistance screening test in the context of an MDR DOTS-plus strategy.

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#### REFERENCES

- Alland, D., G. E. Kalkut, A. R. Moss, R. A. McAdam, J. A. Hahn, W. Bosworth, E. Drucker, and B. R. Bloom. 1994. Transmission of tuberculosis in New York City. An analysis by DNA fingerprinting and conventional epidemiologic methods. *N. Engl. J. Med.* **330**:1710-1716.
- Cockerill, F. R. 1999. Genetic methods for assessing antimicrobial resistance. *Antimicrob. Agents Chemother.* **43**:199-212.
- Dobner, P., G. Bretzel, S. Rüscher-Gerdes, K. Feldmann, M. Rifai, T. Löscher, and H. Rinder. 1997. Geographic variation of the predictive values of genomic mutations associated with streptomycin resistance in *Mycobacterium tuberculosis*. *Mol. Cell Probes* **11**:123-126.
- Farmer, P., and J. Y. Kim. 1998. Community based approaches to the control of multidrug resistant tuberculosis: introducing "DOTS-plus." *Br. Med. J.* **317**:671-674.
- Fleiss, J. L. 1981. Statistical methods for rates and proportions, 2nd ed. John Wiley & Sons, Inc., New York, N.Y.
- Frieden, T. R., L. F. Sherman, K. L. Maw, P. I. Fujiwara, J. T. Crawford, B. Nivin, V. Sharp, D. Hewlett, K. Brudney, D. Alland, and B. N. Kreiswirth. 1996. A multi-institutional outbreak of highly drug-resistant tuberculosis: epidemiology and clinical outcomes. *JAMA* **276**:1229-1235.
- Heifets, L. B., and G. A. Cangelosi. 1999. Drug susceptibility testing of *Mycobacterium tuberculosis*: a neglected problem at the turn of the century. *Int. J. Tuberc. Lung Dis.* **3**:564-581.
- Heym, B., N. Honore, C. Truffot-Pernot, A. Banerjee, C. Schurra, W. R. Jacobs, Jr., J. D. van Embden, J. H. Grosset, and S. T. Cole. 1994. Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study. *Lancet* **344**:293-298.
- Kapur, V., L. L. Li, S. Jordanes, M. R. Hamrick, A. Wanger, B. N. Kreiswirth, and J. M. Musser. 1994. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase  $\beta$  subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. *J. Clin. Microbiol.* **32**:1095-1098.
- Kleeberg, H. H., Z. Blacklock, F. Boulahbal, H. L. David, H. Fink, E. M. S. Gatner, J. Grosset, J. Juhlin, R. Kallich, I. Kawamura, J. O. Kilburn, C. G. Kleeberg, F. Mandler, S. R. Pattyn, K. F. Petersen, H. Reutgen, E. H. Runon, H. Saito, K. H. Schröder, M. F. Stander, I. Szabo, S. Takahashi, S. P. Tripathy, L. Tinka, and B. Vergmann. 1985. A simple method of testing drug susceptibility of *Mycobacterium tuberculosis*: a report of an international collaborative study. *Bull. Int. Union Tuberc.* **60**:147-150.
- Maher, D., P. Chaulet, S. Spinaci, and A. Harries. 1997. Treatment of tuberculosis: guidelines for national programmes, 2nd ed. World Health Organization publication WHO/TB/97.220, p. 77. World Health Organization, Geneva, Switzerland.
- Marttila, H. J., H. Soini, E. Erola, E. Vyshevnskaya, B. I. Vyshevnskiy, T. F. Otten, A. V. Vasilyef, and M. K. Viljanen. 1998. A Ser315Thr substitution in KatG is predominant in genetically heterogeneous multidrug-resistant *Mycobacterium tuberculosis* isolates originating from the St. Petersburg area in Russia. *Antimicrob. Agents Chemother.* **42**:2443-2445.
- Morris, S., G. H. Bai, P. Suffys, L. Portillo-Gomez, M. Fairchok, and D. Rouse. 1995. Molecular mechanisms of multiple drug resistance in clinical isolates of *Mycobacterium tuberculosis*. *J. Infect. Dis.* **171**:954-960.
- Nachamkin, I., C. Kang, and M. P. Weinstein. 1997. Detection of resistance to isoniazid, rifampin, and streptomycin in clinical isolates of *Mycobacterium tuberculosis* by molecular methods. *Clin. Infect. Dis.* **24**:894-900.
- Pablos-Mendez, P., A. Laszlo, F. Bustreo, N. Binkin, D. L. Cohn, C. S. B. Lambregts-van Weezenbeek, S. J. Kim, P. Chaulet, P. Nunn, and M. Ravignione. 1997. Anti-tuberculosis drug resistance in the world. World Health Organization publication WHO/TB/97. 229, p. 36. World Health Organization, Geneva, Switzerland.
- Pozzi, G., M. Meloni, E. Iona, G. Orru, O. F. Thoresen, M. L. Ricci, M. R. Oggioni, L. Fattorini, and G. Orefici. 1999. *rpoB* mutations in multidrug-resistant strains of *Mycobacterium tuberculosis* isolated in Italy. *J. Clin. Microbiol.* **37**:1197-1199.
- Ramaswamy, S., and J. M. Musser. 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber. Lung Dis.* **79**:3-29.
- Telenti, A., N. Honore, C. Bernasconi, J. March, A. Ortega, B. Heym, H. E. Takiff, and S. T. Cole. 1997. Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. *J. Clin. Microbiol.* **35**:719-723.
- Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L. Matter, K. Schopfer, and T. Bodmer. 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* **341**:647-650.
- Van Embden, J. D., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Giequel, P. Hermans, P. Martin, R. McAdam, and T. M. Shinnick. 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J. Clin. Microbiol.* **31**:406-409.
- Van Rie, A., R. Warren, M. Richardson, T. C. Victor, R. P. Gie, D. A. Enarson, N. Beyers, and P. D. van Helden. 1999. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. *N. Engl. J. Med.* **341**:1174-1179.
- Van Rie, A., R. M. Warren, N. Beyers, R. P. Gie, C. N. Classen, M. Richardson, S. L. Sampson, T. C. Victor, and P. D. van Helden. 1999. Transmission of a multidrug-resistant *Mycobacterium tuberculosis* strain among non-institutionalized, human immunodeficiency virus-seronegative patients. *J. Infect. Dis.* **180**:1608-1615.
- Victor, T. C., A. M. Jordaan, A. van Rie, G. D. van der Spuy, M. Richardson, P. D. van Helden, and R. Warren. 1999. Detection of mutations in drug resistance genes of *Mycobacterium tuberculosis* by a dot-blot hybridization strategy. *Tuber. Lung Dis.* **79**:343-348.
- Wengenack, N. L., J. R. Uhl, A. L. St. Amand, A. J. Tomlinson, L. M. Benson, S. Naylor, B. C. Kline, F. R. Cockerill, and F. Rusnak. 1997. Recombinant *Mycobacterium tuberculosis* KatG(S315T) is a competent catalase-peroxidase with reduced activity toward isoniazid. *J. Infect. Dis.* **176**:722-727.
- Weyer, K., P. Groenewald, M. Zwarenstein, and C. J. Lombard. 1995. Tuberculosis drug resistance in the Western Cape. *S. Afr. Med. J.* **85**:499-504.
- Williams, D. L., C. Waguespack, K. Eisenach, J. T. Crawford, F. Portaels, M. Salfinger, C. M. Nolan, C. Abe, V. Sticht-Groh, and T. P. Gillis. 1994. Characterization of rifampin resistance in pathogenic mycobacteria. *Antimicrob. Agents Chemother.* **38**:2380-2386.